AD_____

Award Number: DAMD17-02-1-0431

TITLE: Fatty Acid Synthase Inhibitor Cytotoxicity: Depletion of the Coenzyme-A Pool

PRINCIPAL INVESTIGATOR: Francis P. Kuhajda, M.D.

CONTRACTING ORGANIZATION: The Johns Hopkins University
School of Medicine
Baltimore, Maryland 21205

REPORT DATE: April 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2003	Annual (1 Apr 02 - 31 Mar 03)

4. TITLE AND SUBTITLE
Fatty Acid Synthase Inhibitor Cytotoxicity: Depletion of the Coenzyme-A Pool

5. FUNDING NUMBERS
DAMD17-02-1-0431

6. AUTHOR(S)
Francis P. Kuhajda, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Johns Hopkins University
School of Medicine
Baltimore, Maryland 21205

E-Mail: fkuhajda@jhmi.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Inhibition of fatty acid synthase (FAS) in human cancer cells leads to cytotoxicity without evidence of DNA damage. Based on this and other biochemical observations, attention was focused on the cytoplasm as the site for the origin of C75 cytotoxicity to human cancer cells. In light of recent data that showed a marked increase in malonyl-CoA following FAS inhibition, this grant was focused on coenzyme-A depletion as a key mechanism of action leading to cytotoxicity. While pursuing this line of investigation with exhaustive metabolic labeling studies with [^{14}C]pantothenate during the first 6 months, it became clear that FAS inhibition did not lead to depletion of coenzyme-A. However, it became clear that following FAS inhibition, cancer cells rapidly reduced protein synthesis. This global reduction in protein synthesis closely approximated the endoplasmic reticulum (ER) or unfolded-protein stress response that occurs during apoptosis. Thus, while the focus of the grant remains on studying cytoplasmic events that lead to cancer cell cytotoxicity of FAS inhibition, we have shifted our emphasis from the CoA depletion hypothesis to the ER stress response. We have shown that eukaryotic initiation factor 2 alpha (EIF2α), a key regulator of protein synthesis and the ER stress response, is involved in the cytotoxic mechanism of C75 against human breast cancer cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
EIF2alpha, fatty acid synthase, apoptosis	7
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

4. INTRODUCTION Inhibition of fatty acid synthase (FAS) in human cancer cells leads to cytotoxicity without evidence of DNA damage. Based on this and other biochemical observations, attention was focused on the cytoplasm as the site for the origin of C75 cytotoxicity to human cancer cells. In light of recent data that showed a marked increase in malonyl-CoA following FAS inhibition, this grant was focused on coenzyme-A depletion as a key mechanism of action leading to cytotoxicity. While pursuing this line of investigation with exhaustive metabolic labeling studies with [^{14}C]pantothenate during the first 6 months, it became clear that FAS inhibition did not lead to depletion of coenzyme-A or other small CoA derivatives such as acetyl-CoA. Interestingly, however, it became clear that following FAS inhibition, cancer cells rapidly shut down protein synthesis. This global reduction in protein synthesis was not a result of a lack of high-energy CoA derivatives, but more closely approximated the so-called endoplasmic reticulum (ER) or unfolded-protein stress response that occurs during apoptosis (1). Thus, while the focus of the grant remains on studying cytoplasmic events that lead to cancer cell cytotoxicity of FAS inhibition, we have shifted our emphasis from the CoA depletion hypothesis to implicating the ER stress response. We have shown that eukaryotic initiation factor 2 alpha (EIF2α), a key regulator of protein synthesis and the ER stress response, is involved in the cytotoxic mechanism of C75 against human breast cancer cells.

5. BODY Through these series of experiments, we will outline the relationship FAS inhibition, and subsequent protein synthesis inhibition and the role of eukaryotic initiation factor 2 alpha (EIF2α) in the cytotoxic response.

A. C75 inhibits protein synthesis. During the exploration of CoA metabolism in MCF7 cells, it was noted that C75 significantly inhibits protein synthesis within 4h. Figure 1 shows that protein synthesis is significantly inhibited (p=0.0055) by C75 at 5 μg/ml in MCF7 cells as measured by [^{35}S]methionine incorporation into total protein (error bars represent standard error of the mean). This global reduction in protein synthesis is seen during the apoptosis and is likely mediated by the ER (unfolded protein) stress response. These data led to the investigation of the role of eukaryotic initiation factor 2 alpha (EIF2α), a key protein regulator of the initiation of protein translation.

B. Transfection of MCF-7 cells with a mutant EIF2α. Eukaryotic initiation factor 2 alpha (EIF2α) is central to the regulation of protein translation. When phosphorylated by PRK, EIF2α can induce or promote apoptosis by inhibiting protein synthesis at the level of polypeptide chain initiation. To test the role of EIF2α in C75 mediated apoptosis, we obtained an EIF2α construct from Dr. David Ron's laboratory at the New York University School of Medicine with the an S51A mutation which abrogates its phosphorylation by PRK. Transfection and overexpression of this mutated protein in MCF7 cells will result in constitutively up-regulated protein synthesis and should rescue C75 induced cytotoxicity. Figure 2 shows one example of a stable subclone (#6) of MCF7 cells overexpressing S51A EIF2α compared to the first MCF7 control lane. The Ron lab construct was not designed for stable transfection. Our laboratory removed the EIF2α insert from the Ron lab construct and placed it in a selectable neo vector. This enabled us to select clones via Western blot for up-regulated S51A EIF2α as shown (arrow).

Figure 1.

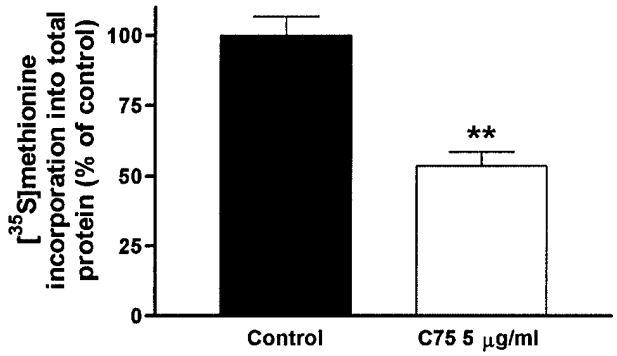
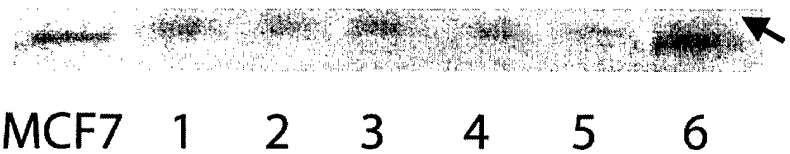
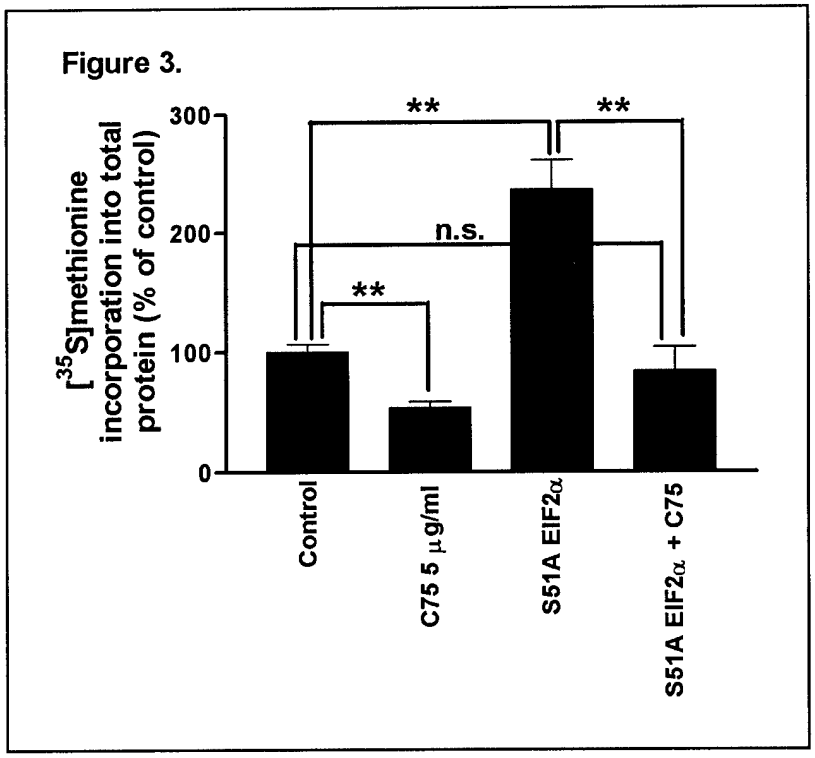


Figure 2.

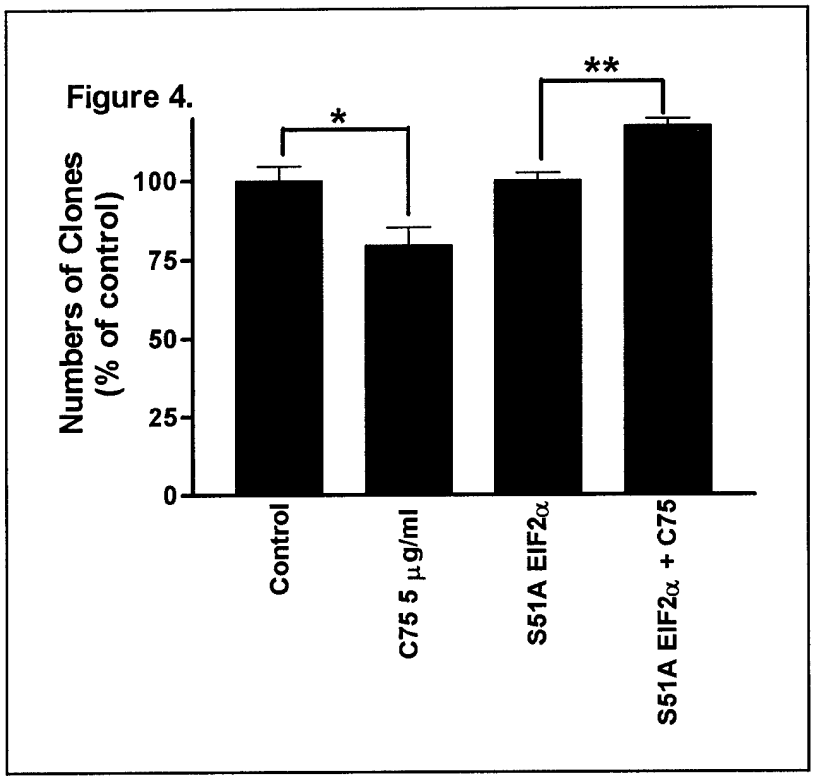


C. The S51A EIF2α mutant increases protein synthesis in MCF7 and C75 treated MCF7 cells. Following stable transfection of the S51A EIF2α into MCF7 cells, protein synthesis was significantly increased over MCF7 controls (Figure 3, p=0.0063). Moreover, although C75 significantly reduced protein synthesis in the S51A EIF2α transfectants (p=0.0089), the C75 treated transfectants had a level of protein synthesis similar to the untreated MCF7 controls. This indicates that the constuitively expressed mutant S51A EIF2α was able to largely rescue protein synthesis from C75 treatment.

Figure 3.



D. S51A EIF2α rescues MCF7 cells from C75 cytotoxicity. If the S51A EIF2α construct can rescue cells from the C75 inhibition of protein synthesis, a rescue from cytotoxicity should also occur. Figure 4 shows the results of a clonogenic assay with MCF7 cells, and S51A EIF2α transfectants and C75 treatment. $4x10^5$ cells were plated in T25 flasks, following overnight incubation, cells were treated with DMSO vehicle or C75 at 5 μg/ml for 6 hours, after tyrpsinization, 800 cells were plated in 60 mm dishes in triplicate. After 8 days, colonies were counted; error bars represent standard error of the mean. C75 treatment of MCF7 cells resulted in a 25% reduction in clonogenicity (p=0.03) while C75 treatment of the S51A EIF2α transfected MCF7 cells resulted in a 17% increase in clonogenicity over controls (p=0.0063). These data demonstrate that the S51A EIF2α construct can abrogate C75 cytotoxicity in MCF7 cells. Since C75 is known to kill MCF7 cells through apoptosis (ref), these data implicate EIF2α and the ER stress response as part of the mechanism of action of C75. Similar results were achieved with simple cell counting assays 48 hours after treating cells with C75 (data not shown).



6. KEY RESEARCH ACCOMPLISHMENTS

A. Depletion of CoA or small CoA derivatives such as malonyl-CoA are not relevant to the mechanism of action of FAS inhibitors such as C75.

B. C75 inhibits protein synthesis as part of its initiation of apoptosis in MCF7 cells.

C. Our laboratory's modification of the construct from Dr. Ron's laboratory at New York University, has led to stably transfected and overexpressed S51A EIF2α in human MCF7 cells.

D. Overexpression of S51A EIF2α in human MCF7 cells leads to increased protein synthesis and abrogation of C75 induced apoptosis.

7. REPORTABLE OUTCOMES Studies with PRK and GADD153 expression in control and S51A EIF2α transfected MCF7 cells are in progress. Once these analyses are complete, we will submit a paper to Cancer Research within the next 3-6 months. We will continue to pursue the role of EIF2α in the mechanism of C75 cytotoxicity in human breast cancer cells.

8. CONCLUSIONS

Fatty acid synthase (FAS) is upregulated in most common human carcinomas. Inhibition of FAS has led to apoptosis in a variety of human cancer cell lines, and C75 has had significant anti-tumor activity in breast, prostate, colon, lung, and mesothelioma xenografts. Understanding the mechanism of cytotoxicity of FAS inhibitors is key to both the basic understanding of the biology and development of FAS as a drug target. C75 triggers the ER stress or unfolded-protein response as part of its apoptotic mechanism in human breast cancer cells. As part of the apoptotic response, C75 also has been shown to inhibit BrdU incorporation into DNA (2) and down-regulate cyclin A and B1 kinase activities (3). We plan to continue exploring the role of EIF2α and its potential to cross-talk with other pathways that are activated as a result of C75 initiation of apoptosis in human breast cancer cells.

9. REFERENCES

1. Clemens, M. J., Bushell, M., Jeffrey, I. W., Pain, V. M., and Morley, S. J. Translation initiation factor modifications and the regulation of protein synthesis in apoptotic cells. Cell Death Differ, *7:* 603-615, 2000.
2. Pizer, E. S., Chrest, F. J., DiGiuseppe, J. A., and Han, W. F. Pharmacological Inhibitors of Mammalian Fatty Acid Synthase Suppress DNA Replication and Induce Apoptosis in Tumor Cell Lines[1]. Cancer Research, *58:* 4611-4615, 1998.
3. Li, J., Gorospe, M., Chrest, F. J., Kumaravel, T. S., Evans, M. K., Han, W. F., and Pizer, E. S. Pharmacological Inhibition of Fatty Acid Synthase Activity Produces Both Cytostatic and Cytotoxic Effects Modulated by p53[1]. Cancer Research, *61:* 1493-1499, 2001.

10. APPENDICES None.